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Short Communication

Temperature and time-dependent effects of delayed blood processing on oxylipin concentrations in human plasma

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ABSTRACT

Background: Oxidized derivatives of polyunsaturated fatty acids, collectively known as oxylipins, are labile bioactive mediators with diverse roles in human physiology and pathology. Oxylipins are increasingly being measured in plasma collected in clinical studies to investigate biological mechanisms and as pharmacodynamic biomarkers for nutrient-based and drug-based interventions. Whole blood is generally stored either on ice or at room temperature prior to processing. However, the potential impacts of delays in processing, and of temperature prior to processing, on oxylipin concentrations are incompletely understood.

Objective: To evaluate the effects of delayed processing of blood samples in a timeframe that is typical of a clinical laboratory setting, using typical storage temperatures, on concentrations of representative unesterified oxylipins measured by liquid chromatography-tandem mass spectrometry.

Design: Whole blood (drawn on three separate occasions from a single person) was collected into 5 mL purple-top potassium-EDTA tubes and stored for 0, 10, 20, 30, 60 or 120 min at room temperature or on wet ice, followed by centrifugation at 4 °C for 10 min with plasma collection. Each sample was run in duplicate, therefore there were six tubes and up to six data points at each time point for each oxylipin at each condition (ice/room temperature). Representative oxylipins derived from arachidonic acid, docosahexaenoic acid, and linoleic acid were quantified by liquid chromatography tandem mass spectrometry. Longitudinal models were used to estimate differences between temperature groups 2 h after blood draw.

Results: We found that most oxylipins measured in human plasma in traditional potassium-EDTA tubes are reasonably stable when stored on ice for up to 2 h prior to processing, with little evidence of auto-oxidation in either condition. By contrast, in whole blood stored at room temperature, substantial time-dependent increases in the 12-lipoxygenase-derived (12-HETE, 14-HDHA) and platelet-derived (thromboxane B2) oxylipins were observed.

Conclusion: These findings suggest that certain plasma oxylipins can be measured with reasonable accuracy despite delayed processing for up to 2 h when blood is stored on ice prior to centrifugation. 12-Lipoxygenase- and platelet-derived oxylipins may be particularly sensitive to post-collection artifact with delayed processing at room temperature. Future studies are needed to determine impacts of duration and temperature of centrifugation on oxylipin concentrations.

1. Introduction

Oxidized derivatives of 18, 20, and 22 carbon polyunsaturated fatty acids, collectively known as oxylipins, are increasingly recognized as bioactive mediators with diverse roles in human physiology and pathology [1–3]. Oxylipins can be divided into three general functional

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classes: (1) autacoids are labile signaling molecules that act locally and are rapidly inactivated or degraded; (2) pathway precursors are intermediates in the biosynthetic pathway leading to generation of more labile, bioactive autacoids; and (3) inactivation products are downstream metabolic derivatives that are often more stable than their autacoid precursors [4]. Oxylipins in these three categories are increasingly being measured in plasma collected in clinical studies to investigate biochemical mechanisms mediating disease [5–8], and to assess for pharmacodynamic biomarkers of nutrient-based and drug-based [9–11] interventions.

Ideally, plasma oxylipin measurements provide an accurate representation of in vivo intravascular levels. However, the labile nature of oxylipins and related compounds presents unique challenges for accurate identification, quantitation, and interpretation of concentrations in human plasma and other tissues [12–15]. There is potential to generate artifact during each step of collection, transport, pre-processing delay, processing, storage, and/or analysis due to: (1) degradation, inactivation, or metabolic conversion, and (2) enzymatic and/or non-enzymatic biosynthesis from precursor fatty acids present in the specimen. The potential for post-collection oxidation may be especially pronounced in polyunsaturated fatty acids containing multiple 1,4-*cis,cis*-pentadiene structures, such as arachidonic acid (AA) and docosahexaenoic acid (DHA) (Fig. 1).

Post-collection artifact could likely be minimized by centrifuging immediately after the collection of whole blood, followed by immediate pipetting, and storing at −80°C until analysis. Ideally, the storage duration will not be excessive and will be uniform among all samples. However, this ideal approach can be challenging to achieve in a clinical setting, where logistical factors often lead to substantial delays before processing and variations in the duration of storage at −80°C before analysis. Following collection, whole blood can be cooled or maintained at room temperature prior to processing, sometimes for periods of several hours. Furthermore, details regarding the time interval between sample collection and processing are rarely reported in manuscripts or discussed as a limitation in papers.

The goal of this pilot project is to evaluate the effects of delayed processing of blood samples in a timeframe that is typical of a clinical setting, using a range of storage temperatures, on concentrations of representative oxylipins, pathway precursors, and inactivation products measured by liquid chromatography tandem mass spectrometry (LC–MS/MS).

2. Methods

The study compares plasma levels of oxylipins in samples that were processed immediately after collection to those that were processed after up to 120 min of storage either on wet ice or at room temperature prior to centrifugation. The protocol was approved by NIH (#03-AG-N322) and written informed consent was obtained for all blood collection procedures. Following an overnight fast, venipuncture of the median cubital vein in the antecubital fossa was performed with an 18-gage butterfly needle, with venous whole blood collected into 5 mL 'purple top' potassium-EDTA (BD Medical) tubes. Whole blood was stored for 0, 10, 20, 30, 60 or 120 min at room temperature or on wet ice (Fig. 2), followed by centrifugation at 4 °C and 1880 g for 10 min.

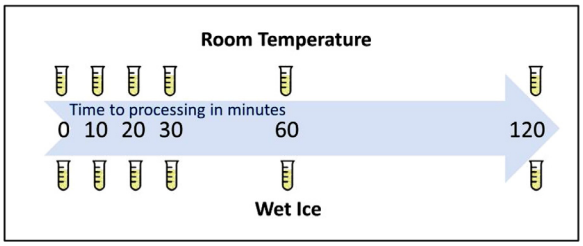

Fig. 2. Sampling timeline used to assess temperature and time-dependent effects of delayed processing on oxylipin concentrations in human plasma.

Following centrifugation, plasma was carefully pipetted into an empty tube with care taken not to disturb the buffy coat. The entire experiment was repeated in three separate rounds on one subject, on three different dates. On each of the three experimental days, each sample was run in duplicate, therefore there were six tubes and up to six data points at each time point for each oxylipin at each condition (wet ice/room temperature).

2.1. Quantitation of oxylipins in plasma

To quantify concentrations of lipid mediators in plasma, lipid extracts were purified using solid phase extraction (SPE) and quantified using LC–MS/MS as previously described [3]. Briefly, SPE of bioactive lipids from biological matrices was performed using Strata X cartridges (33 u, 200 mg/6 mL, Phenomenex, PA). The cartridges were conditioned with 6 mL of methanol, followed by 6 mL of water before samples were extracted. Samples were washed with 6 mL of 10% methanol. The lipids were eluted with 6 mL of methanol into a glass tube containing 10 µL of 30% glycerol in methanol. The eluate was evaporated to dryness under a stream of nitrogen and reconstituted with 40 µL of methanol, and an aliquot (10 µL) was injected into a UPLC (Shimadzu Scientific Instruments, Columbia, MD) system coupled with

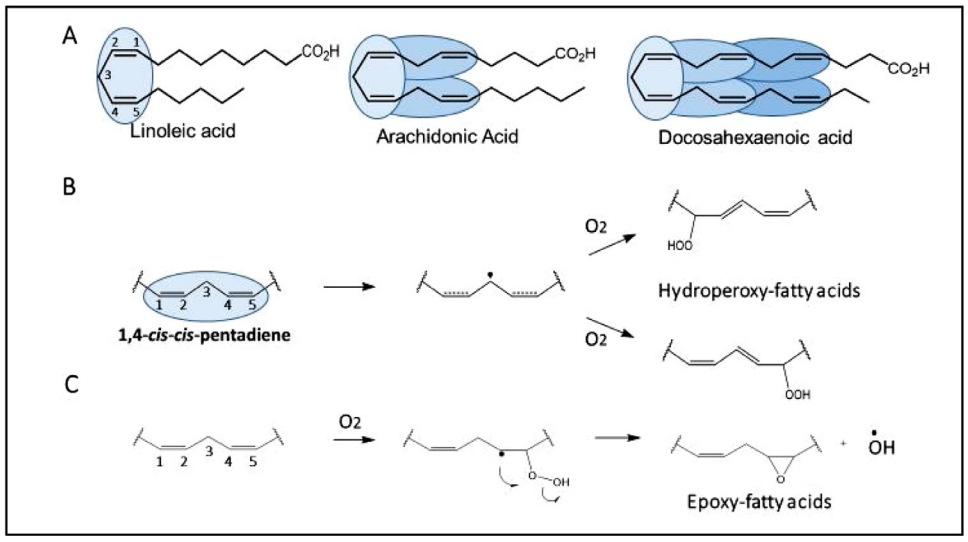

Fig. 1. Oxidation of the 1,4-*cis*-*cis*-pentadiene structures in polyunsaturated fatty acids. (A) Linoleic, arachidonic and docosahexaenoic acid contain one, three and five 1,4-*cis*-*cis*-pentadiene structures, respectively. (B) 1,4-*cis*-*cis*-pentadiene structures are susceptible to enzymatic and free-radical mediated peroxidation, forming hydroperoxy-fatty acids, and (C) epoxy-fatty acids. Hydroperoxy-fatty acids are intermediates in the synthesis of numerous oxylipins.

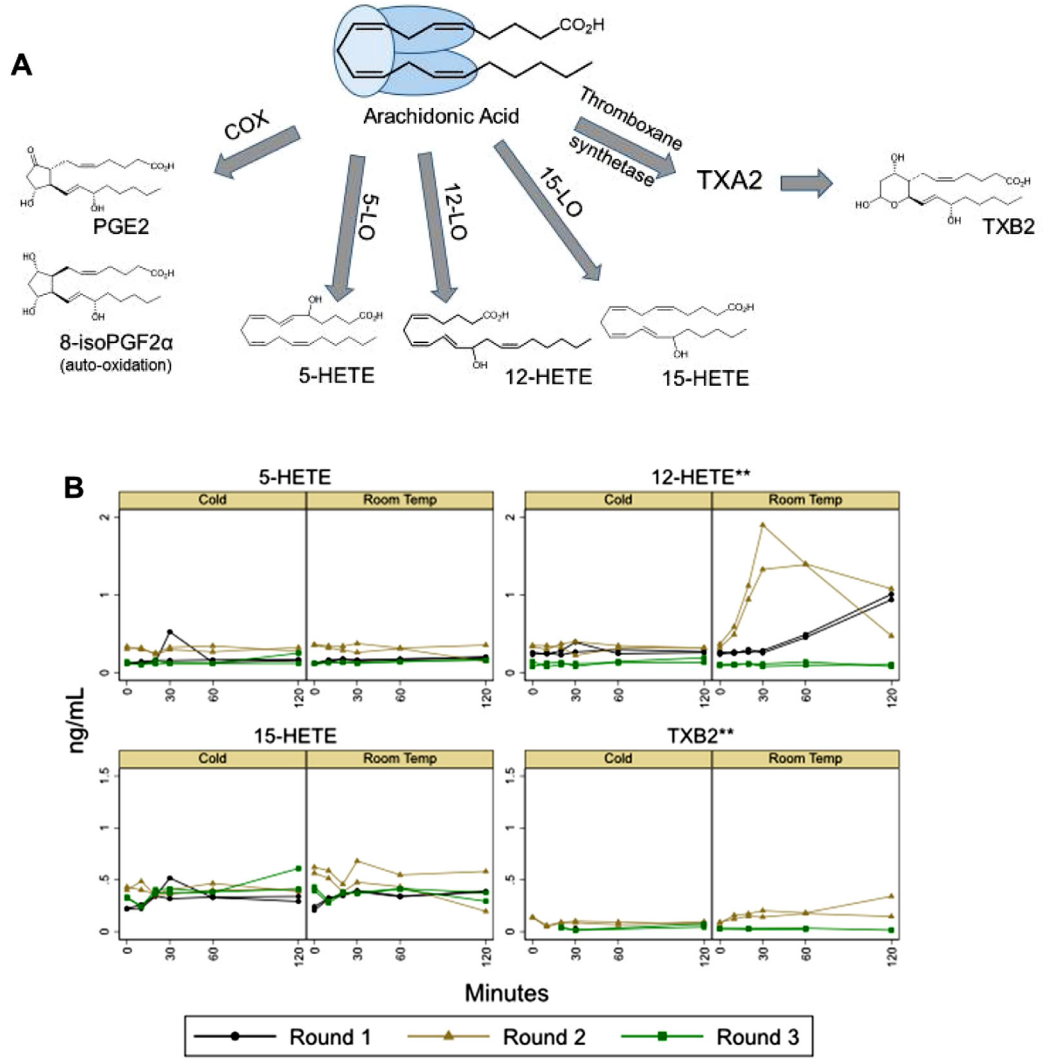

Fig. 3. Arachidonic acid-derived oxylipins: Molecular pathways for synthesis and change from baseline by time and temperature. Panel A shows the molecular pathways involved in enzymatic and non-enzymatic conversion of arachidonic acid (AA) to AA-derived oxylipins. Panel B shows individual data points for plasma concentrations of each of the AA-derived oxylipin measured at baseline and following delayed processing of up to 120 min on wet ice or at room temperature. Rounds with all values below the LOQ are not included in graphs. One half of the LOQ was imputed for values below LOQ. Differences between cold and room temperature at minute 120 min are based on longitudinal mixed models with repeated observations controlling for the data collection round. *P*-values: * < 0.05, ** < 0.01, *** < 0.001.

a Qtrap 5500 (AB SCIEX, USA) for quantitative analysis. Briefly, separation was performed on a ZorBAX RRHD Eclipse Plus C18 column (100 mm × 4 mm; 1.8 μm) (Agilent Corporation, Palo Alto, CA) consisting of (A) 12 mM ammonium acetate solution and acetic acid (100:0.02 v/v) and (B) 12 mM ammonium acetate and was composed of acetonitrile/water/acetic acid (90:10:0.02, v/v/v). The flow rate was 0.5 mL/min. The column oven temperature was set at 30 °C. The elution gradient conditions were as follows: 25–40% B from 0 to 2.0 min, 40–46% B from 2 to 8 min, 46–57% B from 8 to 9 min, 57–66% B from 9 to 20 min, 66–76% B from 20 to 22 min, 76–100% B from 22 to 27 min, held at 100% B from 27 to 33 min, 100–25% B from 33.1 to 35 min. The mass spectrometer was operated in electrospray negative ionization mode using scheduled multiple reaction monitoring (sMRM) acquiring MRM data for each analyte within a retention time window of 90 s. The source parameters were set as follows: ion spray voltage, −4500 V; nebulizer gas (GS1), 65 psi; turbo-gas (GS2), 70 psi; and the turbo ion spray source temperature (TEM), 500 °C. The analytes were quantified using MRM, as previously described [3].

3. Data analysis

To explore and graphically depict the effects of temperature and time on oxylipin concentrations, we plotted individual data points separately by temperature group, for oxylipins that were above the limit of quantitation in at least half of the samples. The limit of quantitation for each oxylipin was defined as a signal-to-noise ratio of > 5. Oxylipin values that were below the limit of quantitation were imputed by substituting one half of the limit of quantitation value for each respective oxylipin. We hypothesized that TXB2, as a platelet derived mediator generated from TXA2, could be particularly sensitive to blood clotting with delayed processing. TXB2 values were above the LOQ in only 33% of samples, and had peaks present in only 53% of samples. Despite this, we included TXB2 in these graphs and our analysis in an exploratory manner. We hypothesized that the differences between the treatments would increase over time and would be highest at the 120-minute measurement. To determine the between-group difference at minute 120, we used longitudinal mixed models for repeated measures controlling for the time, time by group interaction, and measurement round.

4. Results

Temperature and time-dependent effects of delayed processing on the concentrations of sixteen oxylipins derived from AA, DHA and LA that were above the limit of quantitation in half or more of the study samples, plus TXB2, are shown in Figs. 3–5, respectively. Model predicted oxylipin estimates after 120 min of exposure to either wet ice or room temperature and between-group comparisons of wet ice to room temperature are shown in Table 1.

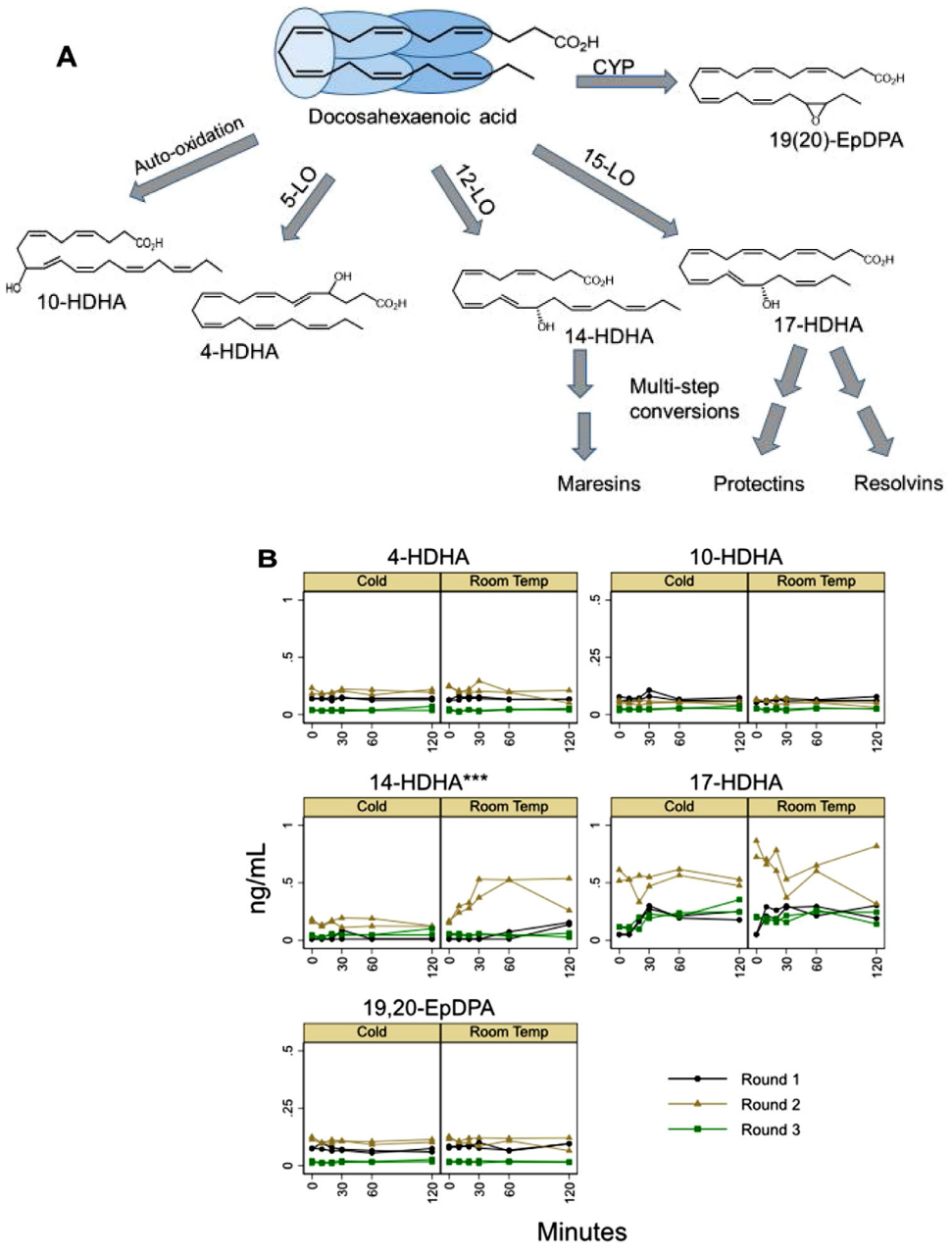

Fig. 4. Docosahexaenoic acid-derived oxylipins: Molecular pathways for synthesis and change from baseline by time and temperature. Panel A shows the molecular pathways involved in enzymatic and non-enzymatic conversion of docosahexaenoic acid (DHA) to DHA-derived oxylipins. Panel B shows individual data points for plasma concentrations of each of the DHA-derived oxylipin measured at baseline and following delayed processing of up to 120 min on wet ice or at room temperature. One half of the LOQ was imputed for values below LOQ. Differences between cold and room temperature at minute 120 min are based on longitudinal mixed models with repeated observations controlling for the data collection round. *P*-values: * < 0.05, ** < 0.01, *** < 0.001.

4.1. AA-derived oxylipins

Biosynthetic pathways for production of six AA-derived oxylipins (PGE2, 8-isoPGF2α, 5-HETE, 12-HETE, 15-HETE, TXB2) are shown in Fig. 3A. Prostaglandin E2 and 8-isoPGF2α were below the limit of quantitation in all samples. Since 8-isoPGF2α is generally considered to be an auto-oxidation product in humans [19], the finding that PGE2 and 8-isoPGF2α remained below the limit of quantitation and did not differ between ice and room temperature, suggests that auto-oxidation likely did not occur to a substantial extent in the conditions of this study. Concentrations of 12-HETE increased substantially over time in samples that had been stored at room temperature prior to processing (Fig. 3A), however these effects were not evident with delayed processing on wet ice. Since 12-HETE can be synthesized by platelet-derived 12-lipoxygenase, this increase suggests that alterations in platelet activation and the coagulation cascade occurred with delayed processing at room temperature [20–23]. Concentrations of TXB2, a more stable inactivation product of labile, bioactive platelet precursor TXA2 [4] (Fig. 3A), also increased over time in samples that had been stored at room temperature prior to processing, however since TXB2 was above the limit of quantitation in only 33% of samples, this finding should be interpreted with some caution. The concentrations of the other two measured AA-derived oxylipins biosynthesized by 5-lipoxygenase (5-HETE) and 15-lipoxygenase (15-HETE), did not change substantially over time and did not differ between the ice and room temperature groups.

4.2. DHA-derived oxylipins

Biosynthetic pathways for production of five DHA-derived oxylipins (4-hydroxy-docohexaenoic acid (4-HDHA), 10-HDHA, 14-HDHA, 17-HDHA and 19,20-epoxy-docosapentaenoic acid (19,20-EpDPA)) are shown in Fig. 4A. Three of these oxylipins (4-HDHA, 10-HDHA, 19,20-EpDPA) appeared stable over time when stored on wet ice or at room temperature. 10-HDHA is generally considered to be an auto-oxidation product in humans because unlike rodents, humans lack 8-lipoxygenase

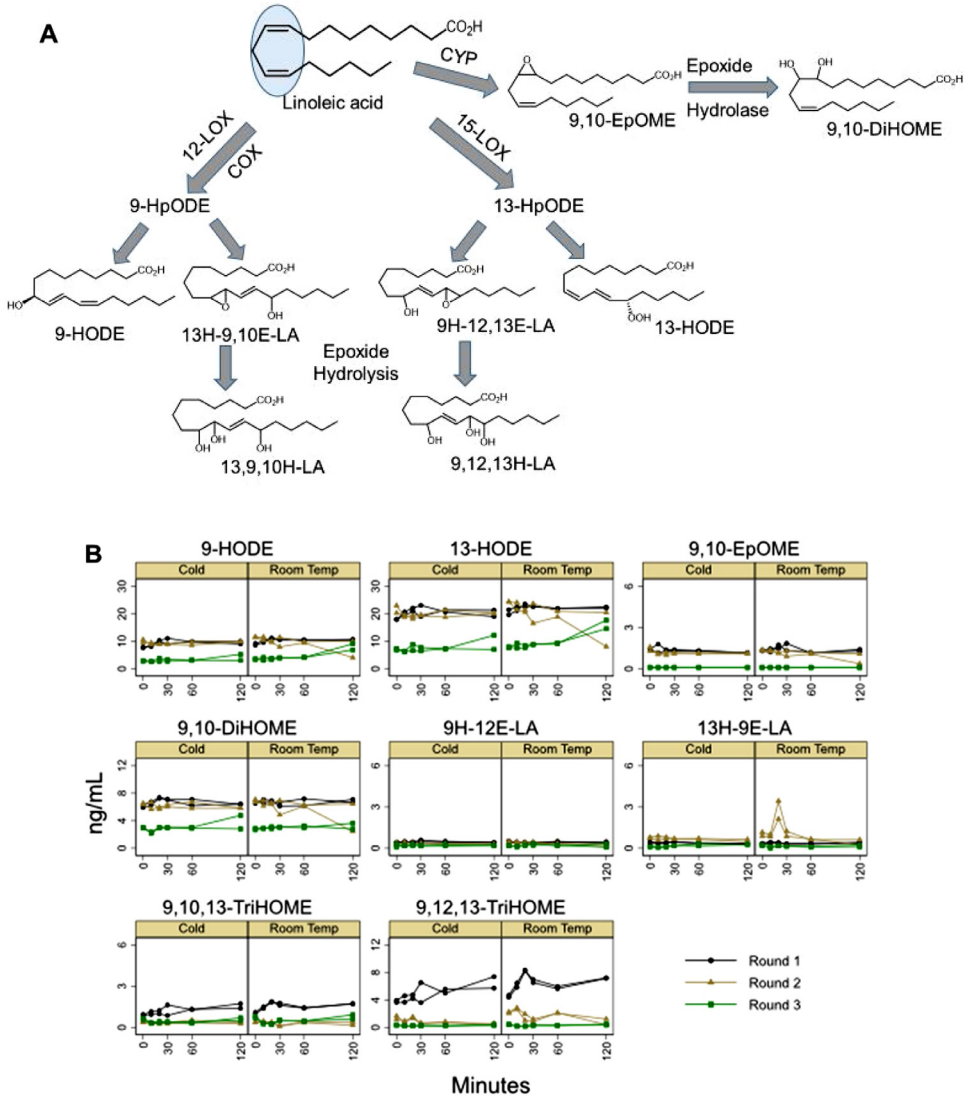

Fig. 5. Linoleic acid-derived oxylipins: molecular pathways for synthesis and change from baseline by time and temperature. Panel A shows the molecular pathways involved in enzymatic and non-enzymatic conversion of linoleic acid (LA) to LA-derived oxylipins. Panel B shows individual data points for plasma concentrations of each of the DHA-derived oxylipin measured at baseline and following delayed processing of up to 120 min on wet ice or at room temperature. One half of the LOQ was imputed for values below LOQ. Differences between cold and room temperature at minute 120 min are based on longitudinal mixed models with repeated observations controlling for the data collection round. *P*-values: * < 0.05, ** < 0.01, *** < 0.001.

Table 1
Model-predicted oxylipin estimates after 120 min of exposure to room temperature or wet ice (ng/ml).

	Wet ice Estimate (95% CI)	Room temperature Estimate (95% CI)	Difference (Room-Wet Ice)[a] Estimate (95% CI)	*P*-value
AA				
5-HETE	0.22 (0.18, 0.26)	0.21 (0.16, 0.25)	−0.01 (−0.07, 0.05)	0.69
12-HETE	0.25 (0.07, 0.43)	0.62 (0.43, 0.80)	0.37 (0.11, 0.62)	0.005
15-HETE	0.41 (0.35, 0.48)	0.36 (0.30, 0.43)	−0.05 (−0.14, 0.04)	0.246
TXB2[b]	0.05 (0.03, 0.08)	0.10 (0.07, 0.12)	0.05 (0.01, 0.08)	0.009
DHA				
4-HDHA	0.13 (0.12, 0.15)	0.11 (0.10, 0.13)	−0.02 (−0.04, 0.00)	0.069
10-HDHA	0.05 (0.04, 0.06)	0.05 (0.04, 0.05)	−0.00 (−0.01, 0.01)	0.466
14-HDHA[b]	0.07 (0.02, 0.12)	0.20 (0.14, 0.25)	0.13 (0.06, 0.20)	0.001
17-HDHA[b]	0.34 (0.27, 0.42)	0.33 (0.26, 0.40)	−0.01 (−0.12, 0.09)	0.787
19,20-EpDPA	0.07 (0.06, 0.07)	0.07 (0.06, 0.08)	0.00 (−0.01, 0.01)	0.713
LA				
9-HODE	7.86 (6.87, 8.86)	8.58 (7.59, 9.57)	0.72 (−0.68, 2.12)	0.316
13-HODE	16.66 (14.70, 18.62)	17.58 (15.63, 19.54)	0.93 (−1.85, 3.70)	0.513
9,10-EpOME[b]	0.79 (0.67, 0.91)	0.72 (0.61, 0.84)	−0.07 (−0.23, 0.10)	0.428
9,10-DiHOME	5.33 (4.85, 5.82)	4.83 (4.34, 5.31)	−0.51 (−1.19, 0.18)	0.149
9-H-12-E-LA	0.30 (0.25, 0.35)	0.28 (0.23, 0.33)	−0.02 (−0.09, 0.04)	0.479
9,10-EpOME[b]	0.39 (0.14, 0.63)	0.32 (0.08, 0.57)	−0.07 (−0.41, 0.28)	0.714
9,10,13-TriHOME	0.83 (0.67, 0.99)	0.92 (0.76, 1.08)	0.09 (−0.14, 0.31)	0.453
9,12,13-TriHOME	2.49 (1.86, 3.12)	2.79 (2.16, 3.41)	0.30 (−0.59, 1.18)	0.513

[a] Based on longitudinal mixed models for repeated measures controlling for the data collection round.
[b] Some observations were below the limit of quantitation (LOQ) and were imputed with ½ of the LOQ.

[24]. Thus, the observation that plasma 10-HDHA did not change over time or differ between ice and room temperature, confirms a lack of substantial auto-oxidation in the conditions of this study. 14-HDHA, a 12-lipoxygenase generated product of DHA, increased substantially in the room temperature samples compared to wet ice, in a similar manner as observed for 12-HETE, suggesting that 12-lipoxygenase activation during clotting is not specific to one substrate. 17-HDHA values varied widely and appeared to be independent of time and temperature.

4.3. LA-derived oxylipins

Proposed biosynthetic pathways for production of eight LA-derived oxylipins are shown in Fig. 5A. Like 12-HETE (derived from AA) and 14-HDHA (derived form DHA), 9-HODE is reported to be a 12-lipoxygenase derivative of LA [25]. However, unlike 12-HETE and 14-HDHA, plasma 9-HODE concentrations were not significantly higher when whole blood was stored at room temperature compared to ice, for up to 2 h. Potential explanations for this discrepancy include that 9-HODE can be synthesized via several other enzymatic and non-enzymatic sources including cyclooxygenases [26,27], 12-lipoxygenase (non-platelet), and free-radical mediated oxidation [28]. Moreover, AA and DHA contain three and five 1,4-*cis,cis*-pentadiene structures respectively (Fig. 1), and therefore are considered to be more susceptible to oxidation compared to LA which contains a single 1,4-*cis,cis*-pentadiene structure.

5. Discussion

Oxylipins hold promise as biomarkers for predicting diseases in observational settings and as pharmacodynamic markers for targeting diet or drug interventions [9,10]. Since plasma oxylipins are derived from more abundant polyunsaturated fatty acids containing 1,4-*cis,cis*-pentadiene structures that are highly susceptible to oxidation, any pre-analytical variable that alters synthesis or degradation could potentially create post-collection artifact in measured metabolites [12,16–18,29], including oxylipins.

In the present study we sought to determine the effect of delayed processing of whole blood samples in a timeframe that is typical of a clinical setting, using typical storage temperatures on concentrations of unesterified oxylipins including pathway precursors, and inactivation products measured by LC–MS/MS. Findings suggest that the concentrations of many oxylipins measured in human plasma using traditional potassium-EDTA tubes are reasonably stable when stored for up to 2 h prior to processing, particularly when stored on wet ice, with little evidence of auto-oxidation products in either condition. The substantial increases observed for 12-lipoxygenase-derived (12-HETE, 14-HDHA) and platelet-derived (TXB2) oxylipins with delayed processing at room temperature indicate that these compounds may be particularly sensitive to post-collection artifact. These findings suggest that certain plasma oxylipins can be measured with reasonable accuracy despite delayed processing for up to 2 h following blood collection, as long as blood is stored on ice prior to centrifugation. The pronounced post-collection artifact in 12-lipoxygenase and platelet-derived oxylipins with delayed processing at room temperature could potentially be due to activation of clotting cascade [20,22,23].

Factors that limit the generalizability of this study include the small sample size, non-independent samples, and the substantial number of samples below the limits of quantitation. Experiments used standard clinical “purple top” potassium EDTA tubes for blood collection and therefore findings are not necessarily generalizable to blood collected using other tubes (i.e. plasma citrate, heparin) or for serum or other blood fractions. Use of heparin tubes has previously been reported to markedly increase oxylipin concentrations [30]. Since serum is generated via activation of the clotting cascade, one could speculate that use of serum separator tubes might alter the production of oxylipins derived from platelets (e.g. 12-HETE, 14-HDHA, thromboxanes) in a similar manner as observed with delayed processing at room temperature in the present study, however this requires experimental confirmation. The finding that oxylipins that were below the limit of quantitation with immediate processing were not increased with delayed processing suggests that the oxylipins profiled in the present study were not synthesized ex vivo. Importantly, some oxylipins have potent bioactions at very low concentrations, and it is possible that certain labile oxylipins were already degraded even with immediate processing. Future studies should examine whether more rapid centrifugation (e.g. 1–2 min) impacts results.

5.1. Summary and conclusions

Plasma samples derived from whole blood that was kept on ice for up to 2 h prior to centrifugation appear suitable for measuring many oxylipins with the caveats noted above. 12-lipoxygenase and platelet-derived oxylipins are sensitive to post-collection artifact with delayed processing at room temperature. Results may have implications for design, implementation, and interpretation of clinical studies measuring oxylipins in plasma. Future studies are needed to determine the impacts of other pre-analytical variables including venipuncture location, needle gauge, blood collection tube type, duration and temperature of centrifugation, and post-processing storage conditions.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.plefa.2019.09.001.

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